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Prokaryotic Expression, Purification, and Production of Polyclonal Antibody Against Novel Human Serum Inhibited Related Protein I (SI1)

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Abstract A novel serum inhibited related gene (SI1) has been cloned in our lab by using mRNA differential display analysis of U251 cells in the presence or absence of serum, the expression of SI1 was dramatically inhibited by the addition of serum to serum starved cells. Previous reports suggested the potential significance of SI1 in regulating the cell cycle. In this study, the plasmid construction, protein expression and purification, as well as the generation of anti-SI1 polyclonal antibody are described. A full-length cDNA of Si1 was inserted in a prokaryotic expression plasmid pET28-b(+) and efficiently expressed in *E. coli* Rosetta (DE3) strain after induction by isopropyl-*b*-D-thiogalactoside. The expressed 6His-tagged SI1 fusion protein was purified by Ni^{+} affinity column and then used to immunize Balb/C mice, and the anti-SI1 polyclonal antibody was purified by protein A column. To determine the sensitivity and specificity of the antibody against SI1, a cell lysate of pEGFP-N2-SI1 plasmid transiently transfected Hela cell was identified by anti-GFP monoclonal antibody and anti-SI1 polyclonal antibody. Both the GFP-SI1 fusion protein and endogenous SI1 protein in Hela cell can be recognized by the anti-SI1 polyclonal antibody. The anti-SI1 polyclonal antibody will provide a useful tool for further characterization of SI1.

Keywords Serum inhibited related gene I (SI1) · Anti-SI1 polyclonal antibody · Prokaryotic expression · Purification · Polyclonal antibody

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Abbreviations

SI1	Serum inhibited related gene/protein 1
GFP	Green fluorescent protein
IPTG	Isopropyl-*b*-D-thiogalactopyranoside
E. coli	*Escherichia coli*
IL-6	Interleukin-6
IL-8	Interleukin-8
INK4a	Inhibitor of CDK4
NCBI	National Center for Biotechnology Information
FBS	Fetal bovine serum
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
PBS	Phosphate-buffered saline
SDS–PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PMSF	Phenyl methane sulphonyl fluoride or phenyl methyl sulphonyl fluoride
MALDI-TOF	Matrix-assisted laser-desorption time-of-flight
OD	Optical density

1 Introduction

Proper cell cycle regulation is key for all eukaryocytes to modulate cell growth and development. A classical model used to study this regulation is how mammalian cells respond to serum [3, 4, 11, 13, 14]. Normally growing cells are arrested in the G_0 phase by serum deprivation and re-enter the cell cycle after the addition of serum [4]. Thus, the identification of those genes induced by serum deprivation, may lead to the discovery of novel tumor suppressors [6]. It has been reported that serum starvation results in attenuation

of the expression of translation initial factors [5] or induction of high level expression of IL-6 and IL-8 to attenuate the expression of Fas-associated death protein (FADD) and caspase-8 gene [1]. Moreover, serum starvation also induces the expression of ARF which is encoded by the alternative transcript of the INK4a gene and inhibits cell growth by stabilization of p53 [7]. Serum inhibited related gene (SI1) was cloned in performing mRNA differential display analysis of U251 cells in the presence or absence of serum, the expression of SI1 was dramatically inhibited by the addition of serum to serum starved cells. Previous studies found that the SI1 protein localized in the nuclei, which would suggest the important function of SI1. Because the sequence of SI1 was aligned in NCBI database and the result showed that there was no high similarity sequence that can presently be found, which indicate that the SI1 would be a novel cell cycle regulation related gene localized in the nuclear [15] and suggested a potential important role in mammalian cell [2, 9, 12]. Several submissions that have a high similarity to SI1 in the Genebank, which indicated that this gene has been of interest to many researchers. Most of the information of this gene currently is from bioinformatic approaches. Lacking a commercial antibody against SI1, studies on biofunctions related to SI1 are limited. Here, we described the cloning and expression of human SI1 gene in *Escherichia coli* (*E. coli*), purification of recombinant proteins, and generation of polyclonal antibody against SI1. The prepared antibody can be useful for the study of expression and distribution of SI1 in various/approaches and elucidate its function.

2 Materials and Methods

2.1 Strains and Reagents

Rosetta plus (DE3) strain was purchased from Invitrogen (USA). pET28b(+) and pEGFP-N2 plasmid were purchased from Novagen and Clontech (USA), while pMD-18T simple vector and all restriction enzymes and the Expand High Fidelity PCR system were purchased from TakaRa (DaLian, China). SP Sepharose and ECL Western blot detection kit were obtained from Beyotime (Jiangsu, China). Hela cell line was maintained in RPMI-1640 medium with ten FBS at 37 °C with 5% CO_2 in our lab. Other reagents were obtained from standard commercial sources and were of reagent grade. All PCR products used for cloning were confirmed by sequencing at Sangon Biotechnology Co., Ltd (Shanghai, China).

2.2 Plasmid Constructions

The SI1 (GeneBank accession no. AY050169) open reading frame fragment was amplified by the Expand High Fidelity PCR system from a T-vector containing full-length SI1 with a pair of gene specific primers (forward: 5′-ggaattccggcgcctgactcgtcg-3′, reverse: 5′-ccgctcgagtcaagagctcaccatgtcccagtgctgg-3′ or forward: 5′-ggaattccggcgcctgactcgtcg-3′, reverse: 5′-ccgctcgagtcaagagctcaccatgtcccagtgctgg-3′) containing the *Bam*HI and *Hin*dIII or *Hin*dIII and *Bam*HI restriction sites, respectively. The reaction was processed with the following programme: 30 s at 98 °C followed by 35 cycles consisting of 30 s at 94 °C, 30 s at 60 °C, 5 min at 72 °C and finally 10 min at 72 °C. The resulting PCR product was *Bam*HI and *Hin*dIII digested, gel extracted and ligated with the kits (Omega Biotechnology Co., USA) in-frame, in a pET28-b(+) and pEGFP-N2 plasmid which were entitled pET28-SI1 and pEGFP-N2-SI1.

2.3 Expression of Recombinant Protein in *E. coli*

pET28-SI1 was transformed into the *Escherichia coli* Rosetta (DE3) strain, the clones were identified by cloning PCR. The positive clone was cultured in 5 mL Luria–Bertani (LB) liquid medium containing both ampicillin (100 mg/mL) and chloramphenicol (60 mg/mL) and grown overnight at 37 °C on a flat rotating incubator, then transferred to 500 mL of fresh medium (with both of the antibiotics) and incubated for another 5 h until the optical density (OD_{600}) of the cultured cells reached 0.6. Expression of the fusion protein was induced with 1 mM isopropyl-*b*-D-thiogalactopyranoside (IPTG) at 37 °C for 5 h. The recombinant protein was analyzed by SDS–PAGE [8].

2.4 Extraction and Purification of 6His-tagged Fusion Proteins

The cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. The pellet was washed with phosphate-buffered saline (PBS) for 2 min × 2 and then suspended (15 mL/g wet weight) in inclusion body wash buffer (PBS, pH 7.3, 1.0 mM EDTA, 1.0 mM PMSF and 1.0 mg/mL lysozyme). The suspension was incubated for 20 min at 4 °C with stirring. Following sonication (30 s × 5 times, pause 2 min), the suspension was centrifuged at 12,000 rpm for 15 min. The clear supernatant (soluble fraction) was collected and the remaining pellet (insoluble fraction) containing inclusion body was resuspensed in an equal volume of lysis buffer. Both soluble and insoluble fractions were then analyzed on 10% SDS–PAGE.

Fusion protein was purified by Ni^+ affinity chromatography (Amersham Biosciences AB, Uppsala, Sweden) according to the manufacturer's protocol. The eluted protein was carefully collected and analyzed by 10% SDS–PAGE. The purified protein was identified by matrix-assisted laser-desorption time-of-flight (MALDI-TOF)

mass spectrometry. (Tianjin Biochip Tech Co., China) and the concentration of the protein was tested by Lowry method.

2.5 Production of Polyclonal Antibody Against the Recombinant SI1 Protein

The purified recombinant protein was used for preparing antibodies in Balb/C mice. Mice was immunized subcutaneously with 500 μg recombinant protein in Freund's complete adjuvant (Sigma, USA) initially. Two booster injections were given with 250 μg recombinant protein each in incomplete Freund's adjuvant at 10 days interval, and the antiserum was collected on the 7 days after the last boost, the antibody titer was determined by ELISA. Then the antiserum was purified by protein A affinity IgG purification kit according the user's guide.

2.6 Cell Culture and Transfection

Hela cells were cultured in 1640 medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 5% CO_2 at 37 °C. Cells were seeded in a 6-well culture plate, and then grown to about 70% density. 12 h before transfection, cells were washed with PBS, and 1 mL/well serum-free 1640 medium was added. pEGFP-N2 with or without a full-length SI1 open reading frame was transfected transiently into Hela cells, respectively, using Lipofectamine 2000 (Invitrogen, USA) according to manufacture's instructions. Each dish was replaced with 2 mL complete 1640 medium after incubation for 4 h, and continue incubated for 48 h. The plates were observed with laser confocal microscope (Zeiss, Germany), the cells were harvested and then lysed in cell lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM *b*-glycerophosphate, 1 mM Na_3VO_4).

2.7 Immuno-blot Assay

Western blotting was performed according to the standard procedure. After 10% SDS–PAGE, the gel was immersed in the transfer buffer (48 mM Tris–HCl, 39 mM glycine, and 20% methanol, pH 9.2), and the proteins were transferred to PVDF membrane. The membrane was incubated for 5 h in 10% fat-free milk at RT for blocking, and being washed 3 min × 10 times with TBS–tween 20 buffer, the membrane was incubated with the anti-SI1 (1:500) or anti-Live Color (1:1500, Colontech, USA) antibody for 6 h at RT. After washing, the membrane was then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10000, Chemicon, USA) at RT for 1 h. The membrane was washed as described above and then analyzed using the ECL (enhanced chemiluminescence) detection system (Beyoutime, China) and the image was exposed to Kodak BioMax X-ray film (Eastman Kodak Co., Rochester, NY) for 1–2 min.

3 Results and Discussion

3.1 Construction of SI1 Expression Vector

Full-length SI1 open reading frame was subcloned into pET18-b(+) and pEGFP-N2 vectors with *Bam*HI and *Hin*dIII sites (Fig. 1a). The recombinant plasmid pET28-SI1 was analysed by restriction enzyme digestion (Fig. 1b) and then confirmed by sequencing.

3.2 Expression of Recombinant Protein

In *E. coli*, the translation rate is slowed when the target protein codon usage differs significantly from the average codon usage of the host organism [10]. Because the SI1 open reading frame was predicted to have serial rare codons. Rosetta (DE3) strain, which has additional tRNAs for rare codons, was employed as the host to improve the expression efficiency of SI1. Expression of the 6His-tagged fusion protein was predicted to encode a recombinant protein with a molecular weight of 90 kDa. Both supernatant and pellet of cell lysate after sonication were analyzed to examine the distribution of expressed recombinant protein in soluble or insoluble fractions. Samples from transformed and non-transformed Rosetta (DE3) were analyzed by SDS–PAGE and stained with Coomassie Brilliant Blue (Fig. 2). Bands of 6His-tagged-SI1 protein with predicted MW of 90 kDa were observed (Fig. 2a), protein was expressed in form of inclusion body.

3.3 Purification and Identification of the Fusion Protein

The 6His-tagged SI1 inclusion body was washed and dissolved for Ni^+ column affinity purification, and the purified protein was analyzed by mass-spectrum (Fig. 2b) and quantified by Lowry method.

3.4 Titer and Specificity Analysis by ELISA and Western Blot

The IgG fraction against SI1 was purified from mice antiserum by $(NH4)_2SO_4$ precipitation and purificated by a protein A Sepharose antibody purification kit (GE Healthcare, USA) and analyzed with SDS–PAGE (Fig. 3a), the titer of the specific antibody was determined by ELISA. The antibody at different dilutions (10- to 10,0000-fold)

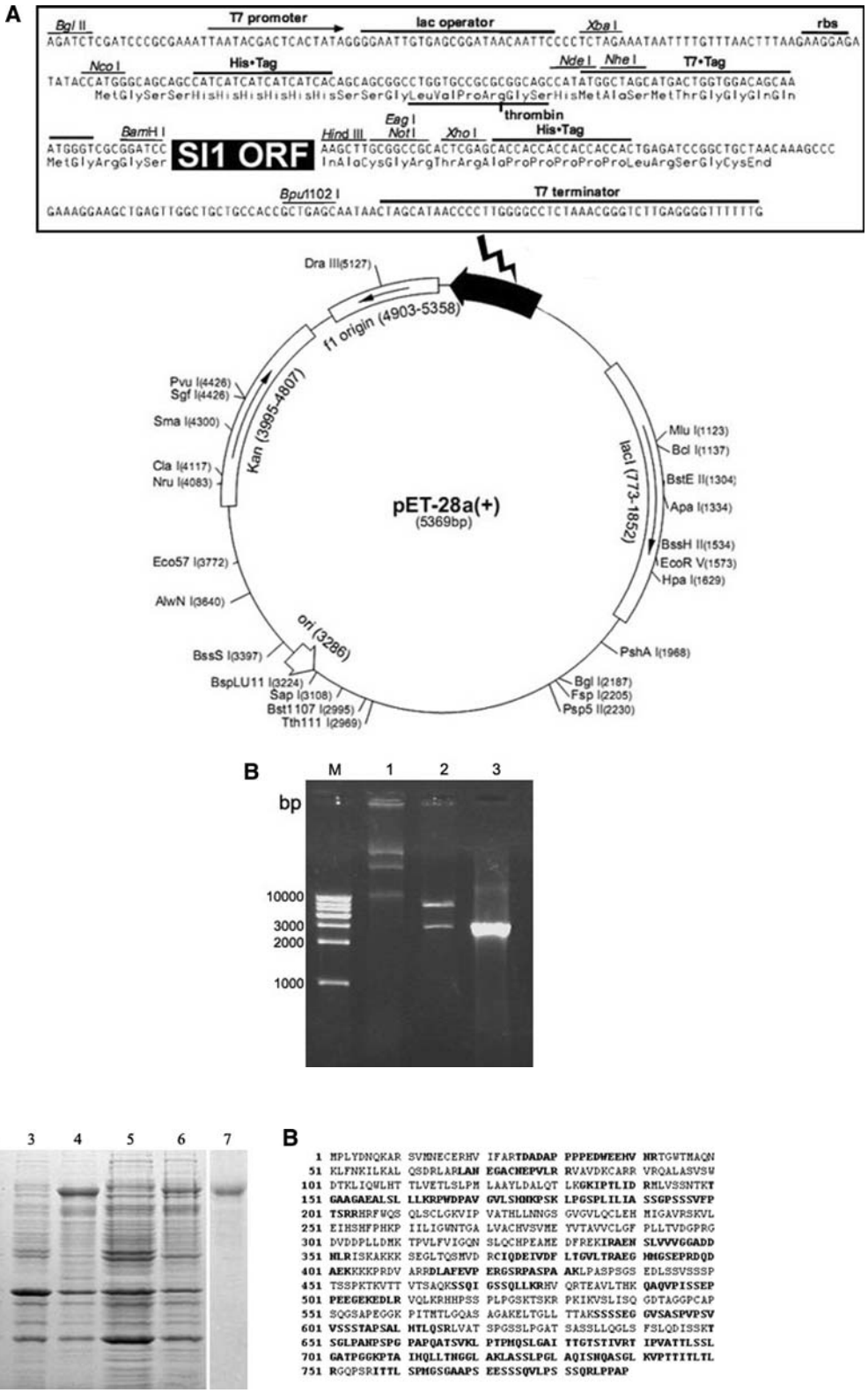

Fig. 1 Structure of recombinant pET28-SI1 prokaryotic expression plasmid and restriction identification. **a** Full length SI1 open reading frame inserted into pET28b(+) vector with *Bam*HI and *Hin*dIII restriction sites. **b** pET28-SI1 plasmid was digested with *Bam*HI and *Hin*dIII enzymes. *M*: 1 kb DNA ladder, *1*: undigested pET28-SI1 plasmid, *2*: *Bam*HI and *Hin*dIII digested pET28-SI1 plasmid, *3*: PCR product amplified from pET28-SI1 plasmid with pET28 sequencing primers

Fig. 2 SI1 protein prokaryotic expression, purification and identification. **a** The pET28-SI1 plasmid transformed Rosetta(DE3) host was induced with 1 mM IPTG, cell lysate was analyzed by SDS–PAGE. *1*: supernatant of cell lysate without induction; *2*: insoluble fraction of cell lysate without induction; *3*: supernatant of cell lysate induced with 1 mM IPTG; *4*: inclusion body of cell lysate induced with 1 mM IPTG; *5*: total cell lysate without IPTG induction; *6*: whole cell lysate induced with 1 mM IPTG; *7*: affinity purified 6His-tagged SI1 protein. **b** Mass-spectrum identification, *bold letters* are the fragments matched with SI1 protein

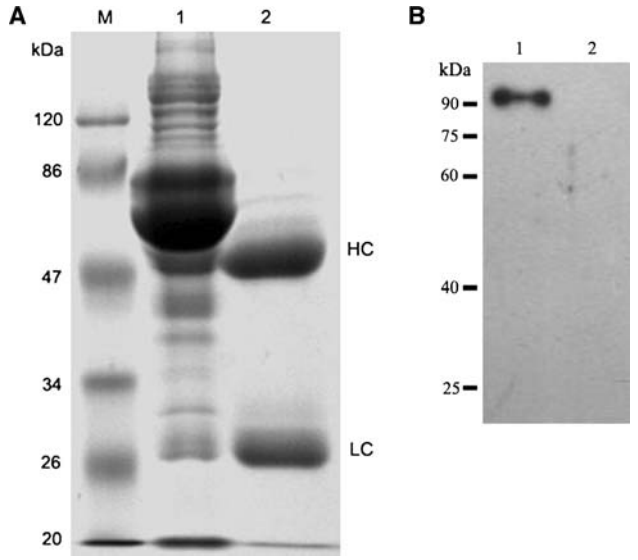

Fig. 3 Purification and identification of anti-SI1 polyclonal antibody. **a** Serum from the mice was separated and purified with protein A column, and analyzed by coomassie blue stained 10% acrylamide gel. *M*: protein molecular standard; *1*: unpurified polyclonal antibody; *2*: purified polyclonal antibody (*HC* = heavy chain, *LC* = light chain). **b** The whole cell lysate of Rosetta (DE3) was analyzed by using purified polyclonal antibody against SI1. *1*: total protein in induced Rosetta (DE3); *2*: total protein in non-induced Rosetta (DE3)

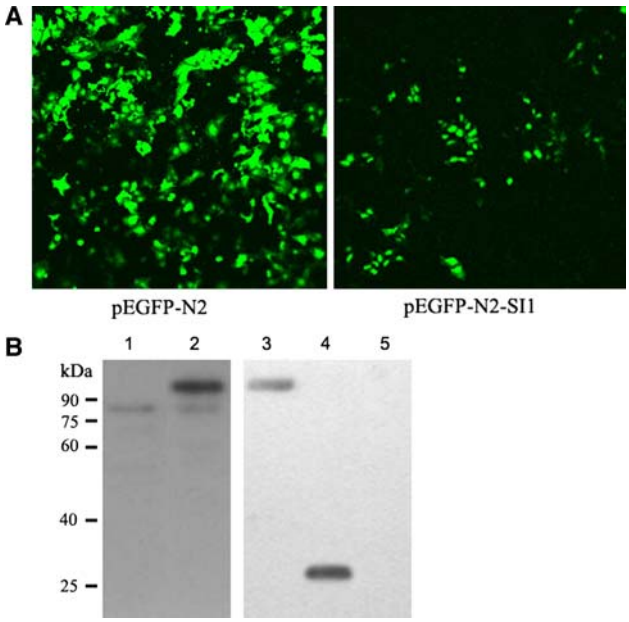

Fig. 4 The obtained anti-SI1 polyclonal antibody specifically recognizes expressed SI1-GFP fusion protein and endogenous SI1 protein in Hela cell. **a** The expression of GFP and SI1-GFP protein was observed under fluorescent microscope. **b** *Lane 1* and *2* were detected with anti-SI1 polyclonal antibody, *lane 3–5* were detected with anti-GFP monoclonal antibody. *1*: Hela cell lysate; *2*: pEGFP-N2-SI1 transfected Hela cell lysate; *3*: pEGFP-N2-SI1 transfected Hela cell lysate; *4*: pEGFP-N2 transfected Hela cell lysate; *5*: non-transfect Hela cell lysate

was reacted with fusion protein, pre-immunized mice serum served as the negative control. The antibody titer was found to be approximately 1:10000. At the same time, negative control did not result in a detectable signal (data not shown).

The activity and specificity of the purified antibody was further determined by western blotting. 6His-tagged SI1 fusion protein was transferred to PVDF membranes and incubated with purified anti-SI1 polyclonal antibody, the result showed a single band in the correct position, which indicated specificity of the produced antibody (Fig. 3b). To determine the validity of the anti-SI1 polyclonal antibody in eukaryotic cell, pEGFP-N2-SI1 and control vector were expressed transiently by transfecting the plasmid into Hela cells. Both the SI1-GFP fusion protein and GFP were observed under the fluorescent microscope (Fig. 4a), which supported the viability of the cell transfection. The cell lysate was probed with anti-SI1 antibody and anti-GFP antibody at a dilution of 1:500 and 1:1500, respectively. An expected 115 kDa band of SI1-GFP fusion protein was detected by anti-SI1 polyclonal antibody and anti-GFP molecular antibody in total lysis of Hela cell transfected with pEGFP-N2-SI1 (Fig. 4b, lanes 2, 3). There was a smaller band (27 kDa) in the lysate of Hela cell transfected with empty vector using anti-GFP antibody (Fig. 4b, lane 4). Western blotting analysis with the polyclonal antibody against the SI1 protein displayed an endogenously 84 kDa band in both pEGFP-N2-SI1 transfection/non-transfection cell lysate (Fig. 4b, lanes 1, 2). The result indicated that Hela cells express SI1 protein endogenously. The purified polyclonal antibody can recognize the SI1 protein with high activity and specificity.

In present study, the endogenous SI1 protein was identified and the size was consistent with the predicted molecular weight. This result proved that the SI1 gene is a functional protein encoding gene.

4 Conclusion

We have efficiently expressed and purified the SI1-6His fusion protein and in addition, we report the first production of a highly specific polyclonal antibody against SI1 and detected the 84 kDa endogenous SI1 protein in the Hela cell. Our result would be an important and useful tool for the future investigation of SI1.

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